

09 | 830502

# HIGH FIDELITY THERMOSTABLE LIGASE AND USES THEREOF

This application claims benefit of U.S. Provisional Patent Application Serial No. 60/106,461 October 30, 1998.

5 The present invention was made with support under National Institutes of Health Grant Nos. GM-41337-09 and PO1-CA65930-02-04). The U.S. Government may have certain rights.

## FIELD OF THE INVENTION

10

The present invention is directed to a high fidelity thermostable ligase and uses thereof.

## BACKGROUND OF THE INVENTION

15

DNA ligases, as an essential component of DNA replication, recombination, and repair systems found from viruses to humans, catalyze the formation of a phosphodiester bond at single-stranded breaks on duplex DNA (Lehman, I.R., *Science*, 186:790-797 (1974)). DNA ligases can be classified into two families based on cofactor dependence. ATP-dependent ligases are found in bacteriophages (Dunn, et al., *J Mol Biol.*, 148(4):303-330 (1981) and Weiss, et al., *Proc Natl Acad Sci USA*, 57(4):1021-1028 (1967)), *Chlorella* virus PBCV-1 (Ho, et al., *J Virol*, 71(3):1931-19374 (1997)), *Vaccinia* virus (Shuman, S., *Biochemistry*, 34(49):16138-161475 (1995)), *Archea* (Kletzin, A., *Nucleic Acids Res*, 20(20):5389-5396 (1992) and Bult, et al., *Science*, 273(5278):1058-1073 (1996)), yeasts (Andaluz, et al., *Yeast*, 12(9):893-8988 (1996), Ramos, et al., *Nucleic Acids Res*, 25(8):1485-1492 (1997), Schar, et al., *Genes Dev*, 11(15):1912-1924 (1997)), mammalian (Tomkinson, et al., *Bioessays*, 19(10):893-901 (1997), Tomkinson, et al., *Mutat Res*, 407(1):1-9 (1998), and Wang, et al., *J Biol Chem*, 269(50):31923-3192811 (1994)), and more recently eubacteria (Cheng, et al., *Nucleic Acids Res*, 25(7):1369-1374 (1997) and Deckert, et al., *Nature*, 392(6674):353-358 (1998)). NAD<sup>+</sup>(i.e. nicotinamide adenine dinucleotide)-dependent ligases, however, are found exclusively

in eubacteria. While some higher eucaryotic organisms may use multiple ATP (i.e. adenosine triphosphate)-dependent ligases to fulfill diverse biological functions, some simple eubacteria genomes could host both an NAD<sup>+</sup>-dependent ligase and an ATP-dependent ligase (Deckert, et al., *Nature*, 392(6674):353-358 (1998) and Fleischmann, et al., *Science*, 269(5223):496-512 (1995)). The origin of the additional ATP-dependent ligases in these genomes remains to be determined.

Although the ATP-dependent ligases and NAD<sup>+</sup>-dependent ligases share little sequence homology, all the ligases investigated so far use the same KXDG motif to form adenylated enzyme intermediate (Tomkinson, et al., *Bioessays*, 19(10):893-901 (1997), Shuman, et al., *Virology*, 211(1):73-83 (1995), and Luo, et al., *Nucleic Acids Res*, 24(15):3079-3085 (1996)). Furthermore, they seem to be organized by similar domains and structural folds ((Doherty, et al., *Nucleic Acids Res*, 24(12):2281-2287 (1996), Subramanya, et al., *Cell*, 85(4):607-615 (1996), and Sekiguchi, et al., *Nucleic Acids Res*, 25(4):727-734 (1997)). The diversity of ligase sequences is not only reflected by their different optimal reaction conditions and kinetic rates, but more importantly by their different specificities toward match and mismatch substrates. Among the viral ATP-dependent ligases, the broad substrate tolerance is represented by the T4 enzyme which seals various mismatches on both the 3' and 5' side of the nick junction (Wu, et al., *Gene*, 76(2):245-254 (1989)).

Vaccinia ligase ligates various mismatches at both 3'-hydroxyl or 5'-phosphate sides with the exception of purine-purine mismatch pairs at the 3'-hydroxyl side (Shuman, S., *Biochemistry*, 34(49):16138-161475 (1995)). Mammalian ATP-dependent ligases show different substrate sensitivity, as ligase I is more sensitive to 3' mismatches than ligase III (Husain, et al., *J Biol Chem*, 270(16):9683-9690 (1995)). Additionally, both ligase I and III tolerate a 3'C/T mismatch more than a 3'G/T mismatch. Little is known about archeal ATP-dependent ligases which may reveal the nature of the progenitor of ATP-dependent ligases. Studies on NAD<sup>+</sup>-dependent DNA ligase from *E. coli*, along with T4 ligase, have contributed immensely to understanding of the basic biochemical pathway of the DNA ligation reaction (Lehman, I.R., *Science*, 186(4166):790-797 (1974) and Rossi, et al., *Nucleic Acids Res*, 25(11):2106-2113 (1997)). Studies on the NAD<sup>+</sup>-dependent ligase from *Thermus thermophilus* HB8 have revealed the highly discriminative power this enzyme possesses (Luo, et al.,

Nucleic Acids Res, 24(15):3071-3078 (1996)). Although mismatches at 5'-phosphate side are tolerated to some degree (5'A/C, 5'A/A, 5'C/A, 5'C/T, 5'G/T, 5'G/A, 5'T/T, 5'T/G), mismatches at the 3'-hydroxyl side essentially abolish nick-closure activity except 3'G/T or 3'T/G mismatch (Luo, et al., Nucleic Acids Res, 24(15):3071-3078 (1996)). Apparently, sequence divergence and subsequent subtle structural variation among DNA ligases underlie an enzyme's recognition preferences toward different mismatched base-pairs.

The study of ligase biochemistry is not only important for understanding its biological functions, but also for developing new technologies. The 10 single nucleotide discrimination observed on DNA ligases has led to the development of ligase-mediated detection techniques (Wu, et al., Gene, 76(2):245-254 (1989), Wu, et al., Genomics, 4(4):560-569 (1989), Landegren, et al., Science, 241(4869):1077-1080 (1988), Landegren, U., Bioessays, 15(11):761-765 (1993), Barany, F., PCR Methods Appl, 1(1):5-16 (1991), and Barany, F., Proc Natl Acad Sci USA, 88(1):189-193 (1991)). Ligase-based linear signal amplification known as LDR (i.e. ligase detection reaction), combined with PCR (i.e. polymerase chain reaction)-based gene specific target amplification, has been proven to be a powerful tool in cancer and disease gene mutation detection (Day, et al., Genomics, 29(1):152-162 (1995)). The 15 PCR/LDR technique relies on two properties of a DNA ligase: (i) specificity and (ii) thermostability. *Tth* (i.e. *Thermus thermophilus* HB8) DNA ligase has been successfully used in LDR and LCR (i.e. ligase chain reaction) due to its highly discriminative nick closure activity toward a perfect match substrate and its thermostability which makes thermocycling possible (Barany, F., PCR Methods Appl, 1(1):5-16 (1991) and Barany, F., Proc Natl Acad Sci USA, 88(1):189-193 (1991)). The 20 To date, one more ligase was cloned and sequenced from *T. Scot.* (i.e. *Thermus scotoductus*) (Thorbjarnardottir, et al., Gene, 161(1):1-6 (1995) and Jonsson, et al., Gene, 151(1-2):177-180 (1994)), but the substrate specificity of this ligase was not determined.

Despite the existence of a number of ligases from different host 30 sources, the need remains to identify additional ligases with greater fidelity. The present invention is directed to achieving this objective as a result of the cloning and

expression of a ligase from *T. sp.* (i.e. *Thermus* species) AK16D and the biochemical characterization of this high fidelity enzyme.

## SUMMARY OF THE INVENTION

5

The present invention is directed to a thermostable ligase having 100 fold higher fidelity than T4 ligase and 6 fold higher fidelity than wild-type *Thermus thermophilus* ligase, when sealing a ligation junction between a pair of oligonucleotide probes hybridized to a target sequence where there is a mismatch with the oligonucleotide probe having its 3' end abutting the ligation junction at the base immediately adjacent the ligation junction.

Another aspect of the present invention is directed to a thermostable ligase having 50 fold higher fidelity than T4 ligase and 5 fold higher fidelity than wild-type *Thermus thermophilus* ligase, when sealing a ligation junction between a pair of oligonucleotide probes hybridized to a target sequence where there is a mismatch with the oligonucleotide probe having its 3' end abutting the ligation junction at the base penultimate to the ligation junction.

Yet another aspect of the present invention is directed to a thermostable ligase having, in the presence of a Mn<sup>2+</sup> cofactor, a 12 fold higher fidelity than wild-type *Thermus thermophilus* ligase, when sealing a ligation junction between a pair of oligonucleotide probes hybridized to a target sequence where there is a mismatch with the oligonucleotide probe having its 3' end abutting the ligation junction at the base immediately adjacent to the ligation junction.

The present invention also relates to a DNA molecule encoding the  
25 thermostable ligase as well as expression systems and host cells containing such DNA  
molecules.

Another aspect of the present invention relates to the use of the thermostable ligase in carrying out a ligase detection reaction process or a ligase chain reaction process.

30 The ligase detection reaction process, involves detecting a target nucleotide sequence which differs from other nucleotide sequences in the sample by one or more single base changes, insertions, deletions, or translocations. This

involves providing a sample potentially containing a target nucleotide sequence which differs from other nucleotide sequences in the sample by one or more single base changes, insertions, deletions, or translocations.

The method further includes providing one or more oligonucleotide

5 probe sets, each characterized by (a) a first oligonucleotide probe having a target specific portion and (b) a second oligonucleotide probe having a target-specific portion. The oligonucleotide probes in a particular set are suitable for hybridization to a target nucleotide sequence which differs from other nucleotide sequences in the sample by one or more single base changes, insertions, deletions, or translocations.

10 The probes are also suitable for ligation together when hybridized adjacent to one another on the target nucleotide sequence, but have a mismatch which interferes with such ligation when hybridized to any other nucleotide sequence present in the sample.

The sample, the one or more oligonucleotide probe sets, and the thermostable ligase are blended to form a ligase detection reaction mixture. The

15 ligase detection reaction mixture is subjected to one or more ligase detection reaction cycles comprising a denaturation treatment and a hybridization treatment. In the denaturation treatment, any hybridized oligonucleotides are separated from the target nucleotide sequence. During the hybridization treatment, the oligonucleotide probe sets hybridize at adjacent positions in a base specific manner to their respective target

20 nucleotide sequences, if present in the sample, and ligate to one another. This forms a ligation product sequence containing the target specific portions connected together with the ligation product sequences for each set being distinguishable from other nucleic acids in the ligase detection reaction mixture. The oligonucleotide probe sets may hybridize to a nucleotide sequence in the sample other than their respective target

25 nucleotide sequences but do not ligate together due to a presence of one or more mismatches and individually separate during the denaturation treatment. The presence of ligation product sequences produced as a result of the target nucleotide sequence being present in the sample is then detected.

In the ligase chain reaction process of the present invention, the

30 presence of a target double stranded nucleic acid formed from first and second complementary target nucleotide sequences is detected in a sample. The target double

stranded nucleic acid differs from other nucleotide sequences by one or more single base changes, insertions, deletions, or translocations.

This method involves providing a sample potentially containing a target double stranded nucleic acid formed from first and second complementary 5 nucleotide sequence. This nucleic acid differs from other nucleotide sequences in the sample by one or more single base changes, insertions, deletions, or translocations.

The method further includes providing a first oligonucleotide probe set, characterized by (a) a first oligonucleotide probe having a target specific portion and (b) a second oligonucleotide probe having a target-specific portion. The 10 oligonucleotide probes in the first set are complementary to the first target nucleotide sequence which differs from other nucleotide sequences in the sample by one or more single base changes, insertions, deletions, or translocations. The probes are also suitable for ligation together when hybridized adjacent to one another on the first target nucleotide sequence, but have a mismatch which interferes with such ligation 15 when hybridized to any other nucleotide sequence present in the sample. The method of the present invention also requires providing a second oligonucleotide probe set, characterized by (a) a third oligonucleotide probe having a target specific portion and (b) a fourth oligonucleotide probe having a target-specific portion. The oligonucleotide probes in the second set are complementary to the second target 20 nucleotide sequence which differs from other nucleotide sequences in the sample by one or more single base changes, insertions, deletions, or translocations. The probes of the second set are suitable for ligation together when hybridized adjacent to one another on the second target nucleotide sequence, but have a mismatch which interferes with such ligation when hybridized to any other nucleotide sequence 25 present in the sample.

The sample, the first and second oligonucleotide probe sets, and the thermostable ligase are blended together to form a ligase chain reaction mixture. The ligase chain reaction mixture is subjected to one or more ligase chain reaction cycles comprising a denaturation treatment and a hybridization treatment. During the 30 denaturation treatment, any hybridized oligonucleotides are separated from the target nucleotide sequences. In the hybridization treatment, the oligonucleotide probe sets hybridize at adjacent positions in a base specific manner to their respective target

nucleotide sequences, if present in the sample. The probes also ligate to one another to form a ligation product sequence containing the target specific portions connected together with the ligation product sequences for each set being distinguishable from other nucleic acids in the ligase chain reaction mixture. The oligonucleotide probe sets may hybridize to nucleotide sequences in the sample other than their respective target nucleotide sequences but do not ligate together due to a presence of one or more mismatches and individually separate during the denaturation treatment. The presence of ligation product sequences produced as a result of the target nucleotide sequence being present in the sample are then detected.

10

## **BRIEF DESCRIPTION OF THE DRAWINGS**

Figures 1A-C show a sequence comparison of *Thermus* DNA ligases. Figure 1A illustrates the evolutionary tree for *Thermus* DNA ligases. Figure 1B is a regional sequence alignment of nine *Thermus* ligases. The aa (i.e. amino acid) sequence of *T. scot* is retrieved from Genebank by accession number 1085749. The adenylation motif KXDG is underlined and the adenylation site is marked by \*. The numbering of aa is based on *Tsp.* AK16D ligase. Figure 1C is a complete amino acid sequence of *Tsp.* AK16D ligase. The adenylation motif KXDG is underlined and the adenylation site <sup>118</sup>K is shown with a (\*) above the residue. The complete sequence of *Tsp.* AK16D ligase gene and partial sequences of six other *Thermus* ligase genes have been deposited with GenBank under accession No. AF092862 for *Tsp.* AK16D, AF092863 for *Thermus aquaticus* YT-1, AF092864 for *Thermus flavus*, AF092865 for *Thermus filiformis* Tok4A2, AF092866 for *Thermus filiformis* Tok6A1, AF092867 for *Tsp.* Vil3, and AF092868 for *Tsp.* SM32.

Figure 2 shows an SDS-PAGE analysis of *Tsp*. AK16D ligase protein. Lane 1, molecular weight markers; Lane 2, uninduced cell lysate; Lane 3, induced cell lysate; Lane 4, supernatant after heating at 70°C; Lane 5, fraction eluted from Hitrap blue column. The SDS-polyacrylamide gel was 0.1% SDS-7.5% polyacrylamide and was stained with Coomassie brilliant blue after electrophoresis. The arrow points to the location of *Tsp*. AK16D ligase.

Figures 3A-C show the effects of salt, pH, and NAD<sup>+</sup> on ligation

activity. *Tsp. AK16D* ligase: closed squares; *Tth* ligase: open squares. Figure 3A reveals the pH effect. Reactions were performed in 20  $\mu$ l mixture containing 200 nM nicked duplex substrate, 12.5 pM *Tth* ligase or *Tsp. AK16D* ligase, 20 mM Tris-HCl (pH values were determined at room temperature), 10 mM MgCl<sub>2</sub>, 100 mM KCl, 10 mM DTT, 1 mM NAD<sup>+</sup> and 20 mg/ml BSA at 65°C for 10 min. Figure 3B shows the salt effect. Reactions were performed in 20  $\mu$ l mixture containing 200 nM nicked duplex substrate, 12.5 pM *Tth* ligase or *Tsp. AK16D* ligase, 20 mM Tris-HCl, pH 8.5 (at room temperature) for *Tth* ligase, pH 8.0 for *Tsp. AK16D* ligase, 10 mM MgCl<sub>2</sub>, indicated amount of KCl, 10 mM DTT, 1 mM NAD<sup>+</sup> and 20 mg/ml BSA at 65°C for 10 min. Figure 3C shows the NAD<sup>+</sup> effect. *Tth* ligation reactions were performed in 20  $\mu$ l mixture containing 200 nM nicked duplex substrate, 12.5 pM *Tth* ligase and indicated concentration of NAD<sup>+</sup>, 20 mM Tris-HCl, pH 8.5, 5 mM MgCl<sub>2</sub>, 100 mM KCl, 10 mM DTT, 1 mM NAD<sup>+</sup> and 20 mg/ml BSA at 65°C for 10 min. *Tsp. AK16D* ligation reaction were performed in 20  $\mu$ l mixture containing 200 nM nicked duplex substrate, 12.5 pM *Tth* ligase and indicated concentration of NAD<sup>+</sup>, 20 mM Tris-HCl, pH 8.5, 5 mM MgCl<sub>2</sub>, 50 mM KCl, 10 mM DTT, 1 mM NAD<sup>+</sup> and 20 mg/ml BSA at 65°C for 10 min.

Figures 4A-B show the divalent cation dependence of *Tsp. AK16D* (stripped bars) and *Tth* (filled bars) ligase activity. Reaction mixtures containing (20  $\mu$ l) 20 nM nicked duplex substrate, 0.5 nM *Tth* ligase or 1 nM *Tsp. AK16D* ligase and 5 mM of indicated divalent cation in the reaction buffers as specified in Figure 3C were incubated at 65°C for 10 min. Figure 4A shows the ligation reactions with different divalent ions as the metal cofactor. Figure 4B shows the chromatogram of a representative GeneScan gel illustrating ligation product and DNA adenylate intermediate. (-): negative control reactions in which ligase was omitted. Co<sup>2+</sup> may have caused precipitation of DNA substrate which resulted in disappearance of the unreacted substrate.

Figures 5A-B shows the time course of *Tth* (Figure 5A) and *Tsp. AK16D* (Figure 5B) ligase activity in the presence of Mg<sup>2+</sup> (open squares) or Mn<sup>2+</sup> (closed squares). Reactions were performed in 100  $\mu$ l mixture containing 20 nM nicked duplex substrate, 0.5 nM *Tth* ligase or 1 nM *Tsp. AK16D* ligase and 5 mM

T05000T-20500500

- 9 -

Mg<sup>2+</sup> or Mn<sup>2+</sup> in the reaction buffers as specified in Figure 3C at 65°C. Aliquots (5  $\mu$ l) were removed at the indicated time and reactions stopped by adding equal volumes of stop solution.

Figures 6A-B show the divalent cation concentration dependence of 5 *Tth* (Figure 6A) and *Tsp*. AK16D (Figure 6B) ligase activity. Mg<sup>2+</sup>(open squares); Mn<sup>2+</sup> (closed squares). Reactions were performed in 20  $\mu$ l mixture containing 20 nM nicked duplex substrate, 0.5 nM *Tth* ligase or 1 nM *Tsp*. AK16D ligase and indicated concentration of Mg<sup>2+</sup> or Mn<sup>2+</sup> in the reaction buffers as specified in Figure 4C at 65°C for 2 min.

Figures 7A-B show the ligation of gapped and inserted substrates. 10 Figure 7A shows the formation of ligated product with gapped and inserted substrates. Reactions were performed in a 20  $\mu$ l mixture containing 12.5 nM nicked duplex substrate, 1.25 pM *Tth* ligase or 12.5 nM *Tsp*. AK16D ligase in the reaction buffer at 65°C for 4 hours. Figure 7B shows the proposed reaction path leads to ligation of 1 nt 15 (i.e. nucleotides) inserted substrate.

## DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a high fidelity thermostable ligase 20 enzyme. This enzyme has the amino acid sequence of SEQ. ID. No. 1 as follows:

MTLEEARRRVNELRDLIRYHNYLYYVDAPEISDAEYDRLLRELKELEERFPELKSP  
DSPTEQVGARPLEATFRPVRHPTRMSLDNAFLSDEVRAFEERIERALGRKGPFLYT  
VERKVDGLSVNLYYEGILVFGATRGDGETGEEVTQNLITIPTIPRRLTGVPDRLEV  
25 RGEVYMPIEAFLRLNQELEEAGERIFKNPRNAAGSLRQKDPRTARRGLRATFYAL  
GLGLEETGLKSQHLLLWLRERGFPVEHGFTRALGAEGVEEVYQAWLKERRKLPFEA  
DGVVVKLDDLALWRELGYTARTPRFALAYKFPAAEKETRLLSVAFQVGRITPVG  
VLEPVFIEGSEVSRTVLHNESFIEELDVRIGDWVLVHKAGGVIPEVLRVLKERRTGE  
EKPIIWPECNCPECGHALIKEGVHRCPNPLCPAKRFEAIRHYASRKAMDIQGLGEKL  
30 IEKLLEKGLVRDVADLYRLKKEDLVNLERMGEKSAENLLRQIEESKGRGLERLLYAL  
GLPGVGEVLARNLALRFGHMDRLLEAGLEDLLEVEVGVGELTARAILNTLKDPEFRDL  
VRRLKEAGVEMEAKEREGEALKGLTFVITGELSVPREEVKALLRRLGAKVTDHSVSRK  
TSFLVVGENPGSKLEKARALGVPTLSEEELYRLIEERTGKDPRALTA

1000007-2050E860

This protein has a molecular weight of 78 to 81 kDa, as measured by SDS-PAGE. For purposes of the present application, the term "thermostable" refers to a DNA ligase which is resistant to inactivation by heat.

The thermostable ligase of the present invention has a 100 fold higher fidelity than T4 ligase and 6 fold higher fidelity than wild-type *Thermus thermophilus* ligase, when sealing a ligation junction between a pair of oligonucleotide probes hybridized to a target sequence where there is a mismatch with the oligonucleotide probe having its 3' end abutting the ligation junction at the base immediately adjacent the ligation junction. This ligase also has a 50 fold higher fidelity than T4 ligase and 5 fold higher fidelity than wild-type *Thermus thermophilus* ligase, when sealing a ligation junction between a pair of oligonucleotide probes hybridized to a target sequence where there is a mismatch with the oligonucleotide probe having its 3' end abutting the ligation junction at the base penultimate to the ligation junction. Finally, the thermostable ligase of the present invention, in the presence of a Mn<sup>2+</sup> cofactor, has a 12 fold higher fidelity than wild-type *Thermus thermophilus* ligase, when sealing a ligation junction between a pair of oligonucleotide probes hybridized to a target sequence where there is a mismatch with the oligonucleotide probe having its 3' end abutting the ligation junction at the base immediately adjacent to the ligation junction. For purposes of the present invention, "fidelity" is defined to mean the ratio of the initial rate of ligating two adjacent probes hybridized to a complementary template with a C-G match at the base of the probe with its 3' end at the ligation junction to the initial rate of ligating two adjacent probes hybridized to a complementary template with a G-T mismatch at the base of the probe with its 3' end at the ligation junction.

The thermostable ligase of the present invention is also characterized by having an arginine adjacent to the active site lysine (i.e. K) in the KXDG motif (where X is any amino acid).

This protein is encoded by a DNA molecule having a nucleotide sequence of SEQ. ID. No. 2 as follows:

30

ATGACCCTAGAGGAGGCCGAGGCGCGTCAACGAACCTCAGGGACCTGATCCGTTAC  
CACAACTACCTCTATTACGTCTTGGACGCCCGAGATCTCCGACGCCGAGTACGAC  
CGGCTCCTTAGGGAGCTTAAGGAGCTGGAGGAGCGCTTCCGAGCTAAAAGCCCC

T000017-20500660

5 GACTCCCCCACGGAACAGGTGGGGCGAGGCCTCTGGAGGCCACCTCCGCCGGT  
CGCCACCCACCGCATGTACTCCCTGGACAACGCCCTTCCTGGACGAGGTGAGG  
GCCTTGAGGAGCGCATAGAGCGGGCCTGGGGCGGAAGGGGCCCTCCTCTACACC  
GTGGAGCGCAAGGTGGACGGCTTCCGTAAACCTCTACTACGAGGAGGGCATCCTC  
5 GTCTTGGGCCACCCGGCGACGGGAGACGGGGAGGAGGTGACCCAGAACCTC  
CTCACCATCCCCACCATTCCCCGCCCTCACGGGCTTCCGGACGCCCTCGAGGTC  
CGGGCGAGGTCTACATGCCATAGAGGCCTCCTCAGGCTCAACCAGGAGCTGGAG  
GAGGCGGGGAGCGCATCTTCAAAAACCCAGGAACGCCGCCGCCGGTCTTGCAG  
10 CAGAAAGACCCAGGGTACGGCCAGGCAGGGCTGAGGGCACCTTACGCCCTG  
GGGCTGGGCCCTGGAGGAAACCGGTTAAAAGCCAGCACGACCTCTCCTATGGCTA  
AGAGAGCGGGCTTCCGTGGAGCACGGCTTACCCGGCCCTGGGGCGGAGGGG  
GTGGAGGAGGTCTACCAGGCCTGGCTCAAGGAGAGGCGGAAGCTTCCCTTGAGGCC  
GACGGGGTGGTGGTCAAGCTGGACGACCTGCCCTCTGGCGGGAGCTGGGTACACC  
15 GCCCGCACCCCCCGCTCGCCCTGCCCTACAAGTTCCGGCGAGGAGAAGGAGACC  
CGCCTCCTCTCCGTGGCCTTCCAGGTGGGGCGGACCGGGCGCATCCCCCGTGGG  
GTTCTGGAGCCCGTCTCATAGAGGGCAGCGAGGTGAGCCGGTCACCCCTCCACAAC  
GAGAGCTTCATTGAGGAGCTGGACGTGCGCATCGCGACTGGTGCTGGTCCACAAG  
GCAGGGCGGGGTGATTCCCGAGGTGCTGAGGGTCTGAAAGAGCGCCGACCGGGAG  
GAGAAGCCCACATCATCTGGCCCAGAGAACTGCCCGAGTGCAGGCCACGCCCTCATCAAG  
20 GAGGGGAAGGTCCACCGCTGCCCAACCCCTTGTGCCCCGCAAGGCCTTGAGGCC  
ATCCGCCACTACGCCCTCCGCAAGGCCATGGACATCCAGGGCTGGGGAGAACGTC  
ATAGAAAAGCTTCTGGAAAAGGCCCTGGTCCGGACGTGGCCGACCTCTACCCCTG  
AAGAAGGAGGACCTGGTGAACCTGGAGCGATGGGGAGAGAGCGCAGAGAACCTC  
25 CTCCGCCAGATAGAGGAGAGCAAGGGCGGGCTGGAGCGCCTCCCTTACGCCCTG  
GGCCTTCCGGGGTGGGGAGGTGCTGGCCCGAACCTGGCCCTCCGCTCGGCCAC  
ATGGACCGCCTCTGGAGGCGGCCATCCTGAATACCTAAAGGACCCGGAGTCCGGGACCTG  
GAGCTACCGCCCGGCCATCCTGAATACCTAAAGGACCCGGAGTCCGGGACCTG  
GTGCGCCGCTGAAGGAGGCCGGGTGGAGATGGAGGCCAAAGAGCGGGAGGGCGAG  
GCCTTGAAGGGGCTCACCTCGTCATCACCGGGAGCTTCCGGCCCGGGAGGAG  
30 GTGAAGGGCCTCTTAGGGGCTTGGGGCAAGGTGACGGACTCGGTGAGGCCAAG  
ACGAGCTTCCGTGGTGGGGAGAACCCGGGAGCAAGCTGGAAAAGGCCCGGCC  
TTGGGGGTCCCCACCCCTGAGCGAGGAGGAGCTACCGCCTCATTGAGGAGAGGAGC  
GGCAAGGACCCAAGGGCCCTCACGGCCTAG

35 Fragments of the above polypeptide or protein are also encompassed by the present invention.

Suitable fragments can be produced by several means. In the first, subclones of the gene encoding the protein of the present invention are produced by conventional molecular genetic manipulation by subcloning gene fragments. The subclones then are expressed *in vitro* or *in vivo* in bacterial cells to yield a smaller protein or peptide that can be tested for ligase activity according to the procedure described below.

As an alternative, fragments of the ligase of the present invention can be produced by digestion of the full-length ligase with proteolytic enzymes like

chymotrypsin or *Staphylococcus* proteinase A, or trypsin. Different proteolytic enzymes are likely to cleave ligase proteins at different sites based on the amino acid sequence of the ligase. Some of the fragments that result from proteolysis may be active ligases.

5 In another approach, based on knowledge of the primary structure of the protein, fragments of the ligase encoding gene may be synthesized by using the PCR technique together with specific sets of primers chosen to represent particular portions of the protein. These then would be cloned into an appropriate vector for increased expression of a truncated peptide or protein.

10 Chemical synthesis can also be used to make suitable fragments. Such a synthesis is carried out using known amino acid sequences for the ligase being produced. Alternatively, subjecting the full length ligase to high temperatures and pressures will produce fragments. These fragments can then be separated by conventional procedures (e.g., chromatography, SDS-PAGE).

15 Variants may also (or alternatively) be modified by, for example, the deletion or addition of amino acids that have minimal influence on the properties, secondary structure and hydropathic nature of the polypeptide. For example, a polypeptide may be conjugated to a signal (or leader) sequence at the N-terminal end of the protein which co-translationally or post-translationally directs transfer of the 20 protein. The polypeptide may also be conjugated to a linker or other sequence for ease of synthesis, purification, or identification of the polypeptide.

25 Suitable DNA molecules are those that hybridize to a DNA molecule comprising a nucleotide sequence of 50 continuous bases of SEQ. ID. No. 2 under stringent conditions characterized by a hybridization buffer comprising 0.9M sodium citrate ("SSC") buffer at a temperature of 37°C and remaining bound when subject to washing with the SSC buffer at 37°C; and preferably in a hybridization buffer comprising 20% formamide in 0.9M saline/0.09M SSC buffer at a temperature of 42°C and remaining bound when subject to washing at 42°C with 0.2x SSC buffer at 42°C.

30 The protein or polypeptide of the present invention is preferably produced in purified form (preferably at least about 80%, more preferably 90%, pure) by conventional techniques. Typically, the protein or polypeptide of the present

invention is secreted into the growth medium of recombinant host cells.

Alternatively, the protein or polypeptide of the present invention is produced but not secreted into growth medium. In such cases, to isolate the protein, the host cell (e.g.,

*E. coli*) carrying a recombinant plasmid is propagated, lysed by sonication, heat, or chemical treatment, and the homogenate is centrifuged to remove bacterial debris.

The supernatant is then subjected to sequential ammonium sulfate precipitation. The fraction containing the polypeptide or protein of the present invention is subjected to gel filtration in an appropriately sized dextran or polyacrylamide column to separate the proteins. If necessary, the protein fraction may be further purified by HPLC.

10 The DNA molecule encoding the ligase of the present invention can be incorporated in cells using conventional recombinant DNA technology. Generally, this involves inserting the DNA molecule into an expression system to which the DNA molecule is heterologous (i.e. not normally present). The heterologous DNA molecule is inserted into the expression system or vector in proper sense orientation and correct reading frame. The vector contains the necessary elements for the transcription and translation of the inserted protein-coding sequences.

U.S. Patent No. 4,237,224 to Cohen and Boyer, which is hereby incorporated by reference, describes the production of expression systems in the form of recombinant plasmids using restriction enzyme cleavage and ligation with DNA ligase. These recombinant plasmids are then introduced by means of transformation and replicated in unicellular cultures including prokaryotic organisms and eucaryotic cells grown in tissue culture.

Recombinant genes may also be introduced into viruses, such as  
vaccinia virus. Recombinant viruses can be generated by transfection of plasmids into  
cells infected with virus.

Suitable vectors include, but are not limited to, the following viral vectors such as lambda vector system gt11, gt WES.tB, Charon 4, and plasmid vectors such as pBR322, pBR325, pACYC177, pACYC1084, pUC8, pUC9, pUC18, pUC19, pLG339, pR290, pKC37, pKC101, SV 40, pBluescript II SK +/- or KS +/- (see "Stratagene Cloning Systems" Catalog (1993) from Stratagene, La Jolla, Calif, which is hereby incorporated by reference), pQE, pIH821, pGEX, pET series (see F.W. Studier et. al., "Use of T7 RNA Polymerase to Direct Expression of Cloned Genes,"

5 Gene Expression Technology vol. 185 (1990), which is hereby incorporated by reference), and any derivatives thereof. Recombinant molecules can be introduced into cells via transformation, particularly transduction, conjugation, mobilization, or electroporation. The DNA sequences are cloned into the vector using standard cloning procedures in the art, as described by Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Springs Laboratory, Cold Springs Harbor, New York (1989), which is hereby incorporated by reference.

10 A variety of host-vector systems may be utilized to express the protein-encoding sequence(s). Primarily, the vector system must be compatible with the host cell used. Host-vector systems include but are not limited to the following: bacteria transformed with bacteriophage DNA, plasmid DNA, or cosmid DNA; microorganisms such as yeast containing yeast vectors; mammalian cell systems infected with virus (e.g., vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (e.g., baculovirus); and plant cells infected by bacteria. The expression 15 elements of these vectors vary in their strength and specificities. Depending upon the host-vector system utilized, any one of a number of suitable transcription and translation elements can be used.

20 Different genetic signals and processing events control many levels of gene expression (e.g., DNA transcription and messenger RNA (mRNA) translation).

25 Transcription of DNA is dependent upon the presence of a promotor which is a DNA sequence that directs the binding of RNA polymerase and thereby promotes mRNA synthesis. The DNA sequences of eucaryotic promotors differ from those of procaryotic promotors. Furthermore, eucaryotic promotors and accompanying genetic signals may not be recognized in or may not function in a procaryotic system, and, further, procaryotic promotors are not recognized and do not function in eucaryotic cells.

30 Similarly, translation of mRNA in procaryotes depends upon the presence of the proper procaryotic signals which differ from those of eucaryotes. Efficient translation of mRNA in procaryotes requires a ribosome binding site called the Shine-Dalgarno ("SD") sequence on the mRNA. This sequence is a short nucleotide sequence of mRNA that is located before the start codon, usually AUG, which encodes the amino-terminal methionine of the protein. The SD sequences are

complementary to the 3'-end of the 16S rRNA (ribosomal RNA) and probably promote binding of mRNA to ribosomes by duplexing with the rRNA to allow correct positioning of the ribosome. For a review on maximizing gene expression, see Roberts and Lauer, Methods in Enzymology, 68:473 (1979), which is hereby 5 incorporated by reference.

Promotors vary in their "strength" (i.e. their ability to promote transcription). For the purposes of expressing a cloned gene, it is desirable to use strong promotors in order to obtain a high level of transcription and, hence, expression of the gene. Depending upon the host cell system utilized, any one of a 10 number of suitable promotors may be used. For instance, when cloning in *E. coli*, its bacteriophages, or plasmids, promotors such as the T7 phage promoter, *lac* promotor, *trp* promotor, *recA* promotor, ribosomal RNA promotor, the  $P_R$  and  $P_L$  promotors of coliphage lambda and others, including but not limited, to *lacUV5*, *ompF*, *bla*, *lpp*, and the like, may be used to direct high levels of transcription of adjacent DNA 15 segments. Additionally, a hybrid *trp-lacUV5* (*tac*) promotor or other *E. coli* promotors produced by recombinant DNA or other synthetic DNA techniques may be used to provide for transcription of the inserted gene.

Bacterial host cell strains and expression vectors may be chosen which inhibit the action of the promotor unless specifically induced. In certain operations, 20 the addition of specific inducers is necessary for efficient transcription of the inserted DNA. For example, the *lac* operon is induced by the addition of lactose or IPTG (isopropylthio-beta-D-galactoside). A variety of other operons, such as *trp*, *pro*, etc., are under different controls.

Specific initiation signals are also required for efficient gene 25 transcription and translation in prokaryotic cells. These transcription and translation initiation signals may vary in "strength" as measured by the quantity of gene specific messenger RNA and protein synthesized, respectively. The DNA expression vector, which contains a promotor, may also contain any combination of various "strong" transcription and/or translation initiation signals. For instance, efficient translation in 30 *E. coli* requires an SD sequence about 7-9 bases 5' to the initiation codon ("ATG") to provide a ribosome binding site. Thus, any SD-ATG combination that can be utilized by host cell ribosomes may be employed. Such combinations include but are not

406007-20500260

limited to the SD-ATG combination from the *cro* gene or the *N* gene of coliphage lambda, or from the *E. coli* tryptophan E, D, C, B or A genes. Additionally, any SD-ATG combination produced by recombinant DNA or other techniques involving incorporation of synthetic nucleotides may be used.

5       Once the isolated DNA molecule encoding the ligase of the present invention has been cloned into an expression system, it is ready to be incorporated into a host cell. Such incorporation can be carried out by the various forms of transformation noted above, depending upon the vector/host cell system. Suitable host cells include, but are not limited to, bacteria, virus, yeast, mammalian cells, 10 insect, plant, and the like.

The present invention is useful in a number of processes where a ligase enzyme is conventionally utilized at high temperatures. Generally, these procedures include the ligase detection reaction and the ligase chain reaction.

15       Both of the ligase detection reaction and ligase chain reaction involve detection of a target sequence and amplification of that sequence at elevated temperatures. In carrying out these procedures, the enzyme is subjected to elevated temperatures but is not degraded due to its thermostable character. The ligase detection reaction and ligase chain reaction procedures are generally described in WO 90/17239 to Barany et. al., F. Barany, et. al., "Cloning, Overexpression, and 20 Nucleotide Sequence of a Thermostable DNA Ligase-Encoding Gene," Gene 109: 1-11 (1991), and F. Barany, et. al., "Genetic Disease Detection and DNA Amplification Using Cloned Thermostable Ligase," Proc. Nat'l Acad. Sci. USA 88: 189-93, the disclosures of which are hereby incorporated by reference.

25       The ligase detection reaction process is useful in detecting in a sample a target nucleotide sequence as described more fully below.

One or more oligonucleotide probe sets are provided for use in conjunction with this method. Each set includes (a) a first oligonucleotide probe having a target-specific portion and (b) a second oligonucleotide probe having a target-specific portion. The oligonucleotide probes in a particular set are suitable for 30 ligation together when hybridized adjacent to one another on a corresponding target nucleotide sequence, but have a mismatch which interferes with such ligation when hybridized to any other nucleotide sequence present in the sample.

The sample, the one or more oligonucleotide probe sets, and the ligase are blended to form a ligase detection reaction mixture. The ligase detection reaction mixture is subjected to one or more ligase detection reaction cycles comprising a denaturation treatment and a hybridization treatment. In the denaturation treatment, 5 any hybridized oligonucleotides are separated from the target nucleotide sequences. The hybridization treatment involves hybridizing the oligonucleotide probe sets at adjacent positions in a base-specific manner to the respective target nucleotide sequences, if present in the sample. The hybridized oligonucleotide probes from each set ligate to one another to form a ligation product sequence containing the target- 10 specific portions connected together. The ligation product sequence for each set is distinguishable from other nucleic acids in the ligase detection reaction mixture. The oligonucleotide probe sets may hybridize to adjacent sequences in the sample other than the respective target nucleotide sequences but do not ligate together due to the presence of one or more mismatches. When hybridized oligonucleotide probes do not 15 ligate, they individually separate during the denaturation treatment.

During the ligase detection reaction phase, the denaturation treatment is carried out at a temperature of 80-105°C, while hybridization takes place at 50- 85°C. Each cycle comprises a denaturation treatment and a thermal hybridization treatment which in total is from about one to five minutes long. Typically, the 20 ligation detection reaction involves repeatedly denaturing and hybridizing for 2 to 50 cycles. The total time for the ligase detection reaction process is 1 to 250 minutes.

The oligonucleotide probe sets can be in the form of ribonucleotides, deoxynucleotides, modified ribonucleotides, modified deoxyribonucleotides, modified phosphate-sugar-backbone oligonucleotides, nucleotide analogs, and mixtures thereof. 25 In one variation, the oligonucleotides of the oligonucleotide probe sets each have a hybridization or melting temperature (i.e.  $T_m$ ) of 66-70°C. These oligonucleotides are 20-28 nucleotides long.

The oligonucleotide probe sets, as noted above, have a reporter label suitable for detection. Useful labels include chromophores, fluorescent moieties, 30 enzymes, antigens, heavy metals, magnetic probes, dyes, phosphorescent groups, radioactive materials, chemiluminescent moieties, and electrochemical detecting moieties.

T050007-2000000000

The product of the ligase detection reaction can be detected in either of two formats. These are fully described in WO 98/03673, to Barany et al., which is hereby incorporated by reference. In one of these formats, ligase detection reaction products are detected by capillary or gel electrophoresis. Alternatively, ligation 5 products can be detected on an array by specific hybridization to a complementary sequence on the array.

The ligation detection reaction mixture may include a carrier DNA, such as salmon sperm DNA.

The hybridization step in the ligase detection reaction, which is 10 preferably a thermal hybridization treatment discriminates between nucleotide sequences based on a distinguishing nucleotide at the ligation junctions. The difference between the target nucleotide sequences can be, for example, a single nucleic acid base difference, a nucleic acid deletion, a nucleic acid insertion, or rearrangement. Such sequence differences involving more than one base can also be 15 detected. Preferably, the oligonucleotide probe sets have substantially the same length so that they hybridize to target nucleotide sequences at substantially similar hybridization conditions. As a result, the process of the present invention is able to detect infectious diseases, genetic diseases, and cancer. It is also useful in environmental monitoring, forensics, and food science.

20 A wide variety of infectious diseases can be detected by the process of the present invention. Typically, these are caused by bacterial, viral, parasite, and fungal infectious agents. The resistance of various infectious agents to drugs can also be determined using the present invention.

Bacterial infectious agents which can be detected by the present 25 invention include *Escherichia coli*, *Salmonella*, *Shigella*, *Klebsiella*, *Pseudomonas*, *Listeria monocytogenes*, *Mycobacterium tuberculosis*, *Mycobacterium avium-intracellulare*, *Yersinia*, *Francisella*, *Pasteurella*, *Brucella*, *Clostridia*, *Bordetella pertussis*, *Bacteroides*, *Staphylococcus aureus*, *Streptococcus pneumonia*, B- 30 Hemolytic strep., *Corynebacteria*, *Legionella*, *Mycoplasma*, *Ureaplasma*, *Chlamydia*, *Neisseria gonorrhoea*, *Neisseria meningitidis*, *Hemophilus influenza*, *Enterococcus faecalis*, *Proteus vulgaris*, *Proteus mirabilis*, *Helicobacter pylori*, *Treponema*

*palladium, Borrelia burgdorferi, Borrelia recurrentis, Rickettsial pathogens, Nocardia, and Actinomycetes.*

Fungal infectious agents which can be detected by the present invention include *Cryptococcus neoformans, Blastomyces dermatitidis, Histoplasma capsulatum, Coccidioides immitis, Paracoccidioides brasiliensis, Candida albicans, Aspergillus fumigatus, Phycomycetes (Rhizopus), Sporothrix schenckii, Chromomycosis, and Maduromycosis.*

Viral infectious agents which can be detected by the present invention include human immunodeficiency virus, human T-cell lymphocytotropic virus, hepatitis viruses (e.g., Hepatitis B Virus and Hepatitis C Virus), Epstein-Barr Virus, cytomegalovirus, human papillomaviruses, orthomyxo viruses, paramyxo viruses, adenoviruses, corona viruses, rhabdo viruses, polio viruses, toga viruses, bunya viruses, arena viruses, rubella viruses, and reo viruses.

Parasitic agents which can be detected by the present invention include *Plasmodium falciparum, Plasmodium malaria, Plasmodium vivax, Plasmodium ovale, Onchocerca volvulus, Leishmania, Trypanosoma spp., Schistosoma spp., Entamoeba histolytica, Cryptosporidium, Giardia spp., Trichomonas spp., Balantidium coli, Wuchereria bancrofti, Toxoplasma spp., Enterobius vermicularis, Ascaris lumbricoides, Trichuris trichiura, Dracunculus medinensis, trematodes, Diphyllobothrium latum, Taenia spp., Pneumocystis carinii, and Necator americanus.*

The present invention is also useful for detection of drug resistance by infectious agents. For example, vancomycin-resistant *Enterococcus faecium*, methicillin-resistant *Staphylococcus aureus*, penicillin-resistant *Streptococcus pneumoniae*, multi-drug resistant *Mycobacterium tuberculosis*, and AZT-resistant human immunodeficiency virus can all be identified with the present invention.

Genetic diseases can also be detected by the process of the present invention. This can be carried out by prenatal or post-natal screening for chromosomal and genetic aberrations or for genetic diseases. Examples of detectable genetic diseases include: 21 hydroxylase deficiency, cystic fibrosis, Fragile X Syndrome, Turner Syndrome, Duchenne Muscular Dystrophy, Down Syndrome or other trisomies, heart disease, single gene diseases, HLA typing, phenylketonuria, sickle cell anemia, Tay-Sachs Disease, thalassemia, Klinefelter Syndrome,

T0500T-0050E050

Huntington Disease, autoimmune diseases, lipidosis, obesity defects, hemophilia, inborn errors of metabolism, and diabetes.

Cancers which can be detected by the process of the present invention generally involve oncogenes, tumor suppressor genes, or genes involved in DNA 5 amplification, replication, recombination, or repair. Examples of these include: BRCA1 gene, p53 gene, APC gene, Her2/Neu amplification, Bcr/Ab1, K-ras gene, and human papillomavirus Types 16 and 18. Various aspects of the present invention can be used to identify amplifications, large deletions as well as point mutations and small deletions/insertions of the above genes in the following common human 10 cancers: leukemia, colon cancer, breast cancer, lung cancer, prostate cancer, brain tumors, central nervous system tumors, bladder tumors, melanomas, liver cancer, osteosarcoma and other bone cancers, testicular and ovarian carcinomas, head and neck tumors, and cervical neoplasms.

In the area of environmental monitoring, the present invention can be 15 used for detection, identification, and monitoring of pathogenic and indigenous microorganisms in natural and engineered ecosystems and microcosms such as in municipal waste water purification systems and water reservoirs or in polluted areas undergoing bioremediation. It is also possible to detect plasmids containing genes that can metabolize xenobiotics, to monitor specific target microorganisms in 20 population dynamic studies, or either to detect, identify, or monitor genetically modified microorganisms in the environment and in industrial plants.

The present invention can also be used in a variety of forensic areas, including for human identification for military personnel and criminal investigation, paternity testing and family relation analysis, HLA compatibility typing, and 25 screening blood, sperm, or transplantation organs for contamination.

In the food and feed industry, the present invention has a wide variety of applications. For example, it can be used for identification and characterization of production organisms such as yeast for production of beer, wine, cheese, yogurt, bread, etc. Another area of use is with regard to quality control and certification of 30 products and processes (e.g., livestock, pasteurization, and meat processing) for contaminants. Other uses include the characterization of plants, bulbs, and seeds for

breeding purposes, identification of the presence of plant-specific pathogens, and detection and identification of veterinary infections.

Desirably, the oligonucleotide probes are suitable for ligation together at a ligation junction when hybridized adjacent to one another on a corresponding 5 target nucleotide sequence due to perfect complementarity at the ligation junction. However, when the oligonucleotide probes in the set are hybridized to any other nucleotide sequence present in the sample, there is a mismatch at a base at the ligation junction which interferes with ligation. Most preferably, the mismatch is at the base 10 at the 3' base at the ligation junction. Alternatively, the mismatch can be at the bases adjacent to bases at the ligation junction.

Before carrying out the ligase detection reaction, in accordance with the present invention, target nucleotide sequences in the sample can be preliminarily amplified. This preferably carried out with polymerase chain reaction. The polymerase chain reaction process is fully described in H. Erlich, et. al, "Recent 15 Advances in the Polymerase Chain Reaction," Science 252: 1643-50 (1991); M. Innis, et. al., PCR Protocols: A Guide to Methods and Applications, Academic Press: New York (1990) and R. Saiki, et. al., "Primer-directed Enzymatic Amplification of DNA with a Thermostable DNA Polymerase," Science 239: 487-91 (1988), which are hereby incorporated by reference.

20 The ligase detection reaction process achieves linear amplification of a target sequence. The ligase chain reaction utilizes essentially the same steps and conditions as the ligase detection reaction to achieve exponential amplification. This greater level of amplification is achieved by utilizing 2 sets of complementary oligonucleotides with each set hybridizing to complementary strands of a target 25 nucleic acid sequence.

In the ligase chain reaction process of the present invention, the presence of a target double stranded nucleic acid formed from first and second complementary target nucleotide sequences is detected in a sample. The target double stranded nucleic acid differs from other nucleotide sequences by one or more single 30 base changes, insertions, deletions, or translocations.

This method involves providing a sample potentially containing a target double stranded nucleic acid formed from first and second complementary

nucleotide sequence. This nucleic acid differs from other nucleotide sequences in the sample by one or more single base changes, insertions, deletions, or translocations.

The method further includes providing a first oligonucleotide probe set, characterized by (a) a first oligonucleotide probe having a target specific portion and (b) a second oligonucleotide probe having a target-specific portion. The oligonucleotide probes in the first set are complementary to the first target nucleotide sequence which differs from other nucleotide sequences in the sample by one or more single base changes, insertions, deletions, or translocations. The probes are also suitable for ligation together when hybridized adjacent to one another on the first target nucleotide sequence, but have a mismatch which interferes with such ligation when hybridized to any other nucleotide sequence present in the sample. The method of the present invention also requires providing a second oligonucleotide probe set, characterized by (a) a third oligonucleotide probe having a target specific portion and (b) a fourth oligonucleotide probe having a target-specific portion. The oligonucleotide probes in the second set are complementary to the second target nucleotide sequence which differs from other nucleotide sequences in the sample by one or more single base changes, insertions, deletions, or translocations. The probes of the second set are suitable for ligation together when hybridized adjacent to one another on the second target nucleotide sequence, but have a mismatch which interferes with such ligation when hybridized to any other nucleotide sequence present in the sample.

The sample, the first and second oligonucleotide probe sets, and the thermostable ligase are blended together to form a ligase chain reaction mixture. The ligase chain reaction mixture is subjected to one or more ligase chain reaction cycles comprising a denaturation treatment and a hybridization treatment. During the denaturation treatment, any hybridized oligonucleotides are separated from the target nucleotide sequences. In the hybridization treatment, the oligonucleotide probe sets hybridize at adjacent positions in a base specific manner to their respective target nucleotide sequences, if present in the sample. The probes also ligate to one another to form a ligation product sequence containing the target specific portions connected together with the ligation product sequences for each set being distinguishable from other nucleic acids in the ligase chain reaction mixture. The oligonucleotide probe

sets may hybridize to nucleotide sequences in the sample other than their respective target nucleotide sequences but do not ligate together due to a presence of one or more mismatches and individually separate during the denaturation treatment. The presence of ligation product sequences produced as a result of the target nucleotide sequence being present in the sample are then detected.

## EXAMPLES

### Example 1 - Reagents, Media, and Strains

10 All routine chemical reagents were purchased from Sigma Chemicals (St. Louis, MO) or Fisher Scientific (Fair Lawn, NJ). Restriction enzymes and T4 DNA ligase were obtained from New England Biolabs (Beverly, MA). Oligonucleotide synthesis reagents, DNA sequencing kits, and PCR kits were 15 obtained from Applied Biosystems Division of Perkin-Elmer Corporation (Foster City, CA). dNTPs, BSA (i.e. bovine serum albumin), ATP were purchased from Boehringer-Mannheim (Indianapolis, IN). *Pfu* DNA polymerase was purchased from Stratagene (La Jolla, CA). *E. coli* strain NovaBlue(DE3)pLysS, and plasmid pET11c were purchased from Novagen, Inc. (Madison, WI). Protein assay kit was from Bio- 20 Rad (Hercules, CA). HiTrap Blue affinity column was from Pharmacia (Piscataway, NJ). LB medium was prepared according to standard formula (Sambrook, et al., (1989) Molecular Cloning-A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1994), which is hereby incorporated by reference). Sonication buffer consisted of 50 mM Tris-HCl, pH 8.0 25 and 1 mM EDTA. TE buffer consisted of 10 mM Tris-HCl, pH 8.0 and 1 mM EDTA. *Tth* DNA ligase and its mutant K294R were purified as previously described (Luo, et al., Nucleic Acids Res, 24(15):3071-3078 (1996), which is hereby incorporated by reference).

30 **Example 2 - Oligonucleotide Synthesis**

Oligonucleotides were synthesized by using a 394 automated DNA synthesizer from Applied Biosystems Division of Perkin-Elmer Corp. PCR and sequencing primers were purified by ethanol precipitation according to instruction

manual. The degenerate sense primer 5'-ATC(T/A)(C/G)CGACGC(C/G)-GA(G/A)TA(T/C)GA-3' (SEQ. ID. No. 3) corresponding to amino acids 32-38 (ISDAEYD) (SEQ. ID. No. 4) in the *T. thermophilus* HB8 DNA ligase gene, and antisense primers 5'-CC(C/G)GT(C/G)C(G/T)-(G/C)CC(G/C)AC(C/T)TG(A/G)AA-5' (SEQ. ID. No. 5) and 5'-GCCTTCTC(C/G/A)A(A/G)(T/C)TTG-(C/G)(A/T)(G/C)CC-3' (SEQ. ID. No. 6) corresponding to amino acids 333-339 (FQVGRTG) (SEQ. ID. No. 7) and 641-647 (GSKLEKA) (SEQ. ID. No. 8) were used to amplify DNA ligase gene fragments from *Thermus* strains. Additional PCR and sequencing primers were synthesized as required. PCR amplification primers for 10 cloning *Tsp. AK16D* DNA ligase gene into pET11c vector were 5'-GCGATTCCATATGACCCTAGAGGAGGCCG-3' (SEQ. ID. No. 9) and 5'-GCGGGATCCGAGGC CTTGGAGAAGCTCTT-3', (SEQ. ID. No. 10) where the *Nde*I and *Bam*HI sites are underlined and the initiation codon in the forward primer is shown in bold. Oligonucleotide substrates for ligation assay were purified on a 15 denaturing sequencing gel (7 M urea/10% polyacrylamide) (Applied Biosystems Inc., The complete guide to evaluating and isolating synthetic oligonucleotides, Applied Biosystems Inc., Foster City, CA (1992)). 5'-phosphorylation of oligonucleotides was achieved during synthesis by using Chemical Phosphorylation Reagent (Glen Research, Sterling, VA). Fluorescent group was attached to a 3'-terminus using 20 Fluorescein CPG column (Glen Research).

### Example 3 - DNA Amplification, Cloning And Sequence Analysis

Genomic DNAs from *Thermus* strains were isolated as previously 25 described (Cao, et al., Gene, 197:205-214 (1997), which is hereby incorporated by reference). PCR amplifications with degenerate and unique primers and inverse PCR on circularized templates were carried out in a GeneAmp PCR System 9700 thermocycler (Applied Biosystems Division of Perkin Elmer) as described (Wetmur, et al., J Biol Chem, 269(41):25928-25935 (1994), which is hereby incorporated by 30 reference). The nucleotide sequences of amplified ligase fragments were directly determined on an ABI 373 sequencer using ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer). Full length *Tsp. AK16D* DNA ligase gene was amplified using PCR amplification primers as described above,

digested with *Nde*I and *Bam*HI, ligated into the cloning vector pET11c treated with the same pair of restriction enzymes, and transformed into *E. coli* strain NovaBlue(DE3)pLysS. Inserts in pET expression vectors were sequenced in both orientations to ensure that the plasmid constructs were free of PCR or ligation error.

5 Nucleic acid and protein sequence analyses were carried out by Clustal method (Higgins, et al., *Comput Appl Biosci*, 5(2):151-153 (1989), which is hereby incorporated by reference) using MegAlign program of DNASTAR (Madison, WI).

10 **Example 4 - Expression and Purification of *Tsp.* AK16D DNA Ligase**

*E. coli* NovaBlue(DE3)pLysS cells containing plasmid pTAK encoding the *Tsp.* AK16D DNA ligase gene from a pET11c construct was propagated overnight at 37°C in LB medium containing 50 µg/ml ampicillin, 25 µg/ml chloramphenicol, and 0.2% glucose. Overnight cultures were diluted 100-fold into 15 the same medium, grown until the optical density of the culture reached 0.5 at 600 nm, then induced by the addition of IPTG to a final concentration of 1 mM, and grown for an additional 4 hrs under the same conditions. Cells were collected by centrifugation, frozen/thawed at -20°C/23°C, disrupted by sonication, and clarified by centrifugation as previously described (Wetmur, et al., *J Biol Chem*, 269(41):25928-20 25935 (1994), which is hereby incorporated by reference). The resulting supernatants were heated at 70°C for 15 min to denature thermolabile *E. coli* proteins, placed on ice for 30 min to aggregate the denatured proteins, and cleared of denatured proteins by microcentrifugation for 15 min at 4°C. The partially pure DNA ligase was further purified by chromatography using 1 ml HiTrap Blue affinity column. Briefly, the 25 column containing *Tsp.* AK16D DNA ligase was washed extensively with TE buffer (pH 7.8) containing 0.1 M NaOAc, and the ligase was eluted with TE buffer (pH 7.8) containing 2 M NaCl. After dialysis against TE buffer (pH 8.0) containing 0.2 M KCl and concentration using Centricon-30 (Amicon), protein concentration was assayed by the Bradford method with reagents supplied by Bio-Rad protein assay kit. The 30 amount of protein was determined using BSA as the standard. The purity of the ligase was verified through 7.5% SDS (i.e. sodium dodecyl sulfate)-PAGE (i.e. polyacrylamide gel electrophoresis) analysis followed by visualizing the overloaded gel with routine Coomassie Brilliant Blue R staining.

P0500072000000000

**Example 5 - Substrates And Ligation Assay**

The oligonucleotide perfect match substrate was formed by annealing

5 two short oligonucleotides (33-mer for LP3'C (SEQ. ID. No. 11) and 30-mer for Com3F (SEQ. ID. No. 12)) with a 59-mer complementary oligonucleotide (Glg). Oligonucleotides LP3'C and Glg (SEQ. ID. No. 14) were in 1.5-fold excess so that the all the 3' Fam labeled Com3F represented nicked substrates (see Luo, et al., *Nucleic Acids Res*, 24(15):3071-3078 (1996), which is hereby incorporated by reference).

10 The T/G mismatch substrate was formed by annealing LP3'T (SEQ. ID. No. 13), which introduced a single base-pair mismatch at the 3'-end of the nick junction, along with Com 3'F to the complementary strand (Glg). The nicked DNA duplex substrates were formed by denaturing DNA probes at 94°C for 2 min followed by re-annealing at 65°C for 2 min in ligation buffer. The sequences of the oligonucleotides were

15 listed below (p represents 5' phosphate group):

|       |   |  |
|-------|---|--|
| LP3'T | 5' - CCCTGTTCCAGCGTCTGCGGTGTTGCGTT              | pAGTTGTCATAGTTGATCCTCTAGTCTGGG - Fam - 3' Com3 |
| LP3'C | 5' - AAAACCCCTGTTCCAGCGTCTGCGGTGTTGCGTC         |  |
| Glg   | 3' - GGGACAAGGTCGCAGACGCCACAAACGAGTCAACAGTATCAA | ACTAGGAGATCAGACCC - 5'                         |

Ligation mixtures (20  $\mu$ l) containing indicated amount of DNA ligase

20 and match or mismatch substrate in the ligase buffer (20 mM Tris-HCl, pH 7.6 at room temperature; 10 mM MgCl<sub>2</sub>; 100 mM KCl; 10 mM DTT (i.e. dithiothreitol); 1 mM NAD<sup>+</sup>; and 20 mg/ml BSA) were incubated at 65°C for a predetermined time. Reactions were terminated by the addition of an equal volume of stop solution (i.e. 50 mM EDTA, 80% formamide, and 1% Blue Dextran). Samples (5  $\mu$ l) were

25 electrophoresed through an 8 M urea-10% polyacrylamide GeneScan gel according to instructional manual (Perkin Elmer). The unreacted substrates were represented by the 30-mer com3F and products were represented by a ligated 63-mer in the case of the match substrate. Both the remaining substrates and ligated products were quantified using GeneScan analysis software 672 (version 2.0, Perkin Elmer).

**Example 6 - Steady State Kinetics**

Steady state kinetic constants were determined by measuring initial rates of the ligation reaction at a given substrate concentration (nicked DNA duplex 5 substrate concentration ranging from 25-400 nM) and a given ligase concentration (12.5 pM for both *Tth* and *Tsp. AK16D*) in 100  $\mu$ l reaction volume at 65°C. A 5  $\mu$ l aliquot was removed at 0, 2, 4, 6, 8, 10 min, and mixed with 5  $\mu$ l of stop solution. The 10 remaining substrate was separated from ligated product by GeneScan gel as described above. Initial rates of the ligation reactions were calculated from the generation of ligated product over time. The  $K_m$  and  $k_{cat}$  values were determined using computer software Ultrafit (Biosoft, Ferguson, MO).

**Example 7 - Sequence Analysis Of Seven *Thermus* Ligase Genes**

15 Amino acid sequence alignment of five Gram negative bacterial NAD<sup>+</sup>-dependent DNA ligases indicates that *Tth* ligase is 93% identical to *Thermus scotoductus* ligase, 49% to *Rhodothermus marinus* ligase, 48% to *E. coli* ligase, and 38% to *Zymomonas mobilis* based on sequence data retrieved from GeneBank. Degenerate primers corresponding to highly conserved regions of these ligases were 20 used to amplify fragments of ligase genes from seven *Thermus* strains which represent a worldwide collection: *Thermus flavus* from Japan (SEQ. ID. No. 16), *Thermus aquaticus* YT-1 (SEQ. ID. No. 15) and *Thermus sp. AK16D* from Yellowstone National Park in the United States, *Thermus filiformis* Tok4A2 (SEQ. ID. No. 17) and *Thermus filiformis* Tok6A1 (SEQ. ID. No. 18) from New Zealand, *Thermus sp. SM32* 25 (SEQ. ID. No. 19) from Azores, and *Thermus sp. Vil3* (SEQ. ID. No. 20) from Portugal. The sequences of amplified ligase fragments ranging from 1.4 to 1.6 kb were determined by directly sequencing the PCR products using an ABI 373 automated sequencer. *Thermus* ligases, in general, were highly conserved during evolution as demonstrated by 85%-98% sequence identity. In contrast, the amino acid 30 sequences of the restriction endonuclease *TaqI* and its isoschizomers from the identical strains show only 50-70% aa identities (Cao, et al., *Gene*, 197:205-214 (1997), which is hereby incorporated by reference). *Thermus* ligases in general show 30-40% sequence identities as compared with DNA ligases from other bacteria. The

sequence divergence is slightly higher among the different geographic groups than within the same group, which may reflect random drift or adaptation to their respective local environments (Figure 1). *Thermus flavus*, *Thermus filiformis* Tok4A2, *Thermus filiformis* Tok6A1, *Thermus sp.* SM32, *Thermus sp.* Vil3, *Thermus aquaticus* YT-1, and *Thermus sp.* AK16D (SEQ. ID. No. 14) ligases shared 98.2%, 5 89.9%, 89.5%, 89.8%, 88.3%, 88.2%, 88.1% with *Thermus thermophilus* HB8 DNA ligase, respectively. The adenylation site of the enzymes (<sup>118</sup>KXDG where X is in general a hydrophobic residue), as identified by site-directed mutagenesis of *Tth* DNA ligase, is completely identical among all *Thermus* ligases, furthermore, the flanking 10 sequences of the adenylation motif are also identical except *Tsp.* AK16D in which the aa residues <sup>117</sup>H before the <sup>118</sup>K is substituted by an <sup>118</sup>R (Figure 1B). In non-*Thermus* NAD<sup>+</sup>-dependent ligases discovered to date, the corresponding position is either a Pro or a Leu. The two isolates from Japan can be distinguished from the other *Thermus* strains by a 3-aa-insertion at position 234.

15

**Example 8 - Cloning, Expression And Purification Of DNA Ligase From *Tsp.* AK16D**

To maximize the chance of finding a *Thermus* ligase with novel 20 properties, *Tsp.* AK16D ligase was chosen which showed the least sequence identity as compared with *T. thermophilus* ligase. To obtain the complete sequence of the ORF (i.e. open reading frame), the fragments of the N- and C-terminus of the gene were amplified by inverse PCR and were subject to direct sequencing. The complete 25 ORF of the *Thermus sp.* AK16D ligase gene consists of 674 amino acids, as compared to 676 aa for *Tth* ligase and 674 aa for *T. scot* ligase (Figure 1C). The full-length *Thermus sp.* AK16D ligase gene was PCR amplified using *Pfu* polymerase and cloned into expression plasmid pET11c (Novagen). The integrity of the insert containing the ligase gene was verified by DNA sequencing. The pET11c plasmid expressing *Tsp.* AK16D ligase was transformed into competent *E. coli* cells NovaBlue(DE3)pLysS. 30 Production of ligases was induced by adding IPTG to 1 mM final concentration. *Tsp.* AK16D ligase protein was expressed to approximately 10% of total cellular proteins (Figure 2, lane 3). Heating at 70°C for 15 minutes denatured most of *E. coli* proteins while leaving the thermostable ligases as the dominant band (Figure 2, lane 4). A

090001-200000

cibacron blue based affinity chromatography (Pharmacia) further removed residual *E. coli* proteins and nucleic acids, yielding apparently homogenous *Tsp.* AK16D ligase protein as judged by Coomassie staining (Figure 2, lane 5).

5 **Example 9 - Salt, pH, and NAD<sup>+</sup> Dependence Of The Ligation Reaction**

Figure 3A depicts the pH dependence of ligase activity of *Tth* and *Tsp.* AK16D ligase proteins. The shape of the pH dependence curves of *Tth* ligase and *Tsp.* AK16D ligase is essentially superimposable. The optimal pH is 8.5 for both *Tth* ligase and *Tsp.* AK16D ligase with greater than 80% activity observed between pH 10 7.8 and 9.5. The identity of pH effect suggests that both of the ligases possess similar local environment at their catalytic center, which is in agreement with the degree of sequence conservation between the two ligases. Figure 3B depicts the salt 15 concentration dependence of ligase activity of *Tth* and *Tsp.* AK16D ligase proteins. The optimum KCl concentration for *Tth* ligase and *Tsp.* AK16D ligase are 100 and 50 mM, respectively. Figure 3C depicts the NAD<sup>+</sup> concentration dependence of ligase 20 activity of *Tth* and *Tsp.* AK16D proteins. The optimum NAD<sup>+</sup> concentration is 1 mM for both *Tth* ligase and *Tsp.* AK16D ligase. The similarity of the NAD profiles is in keeping with the highly conserved nature of the N-terminal domain of the ligases which is involved in NAD<sup>+</sup> binding.

**Example 10 - Effects Of Divalent Metals On The Ligation Reaction**

Divalent metal ion is indispensable for each of the three steps in a 25 ligation reaction: (i) adenylation of a lysine residue in the adenylation motif KXDG; (ii) transfer of the adenylate to the 5' phosphate to form a DNA-adenylate intermediate; and (iii) formation of the phosphodiester bond with the release of adenine monophosphate (AMP). In general, Mg<sup>2+</sup> is the preferred metal ion for 30 both ATP-dependent and NAD<sup>+</sup>-dependent ligases. Mg<sup>2+</sup> was substituted with alkaline earth metal ion Ca<sup>2+</sup> and commonly studied period 4 transition metal ions. *Tth* and *Tsp.* AK16D ligases could use Mn<sup>2+</sup> as an alternative metal cofactor to support ligation activity (Figure 4). Both enzymes were less active with Ca<sup>2+</sup>, while Co<sup>2+</sup>, Ni<sup>2+</sup>, Cu<sup>2+</sup>, and Zn<sup>2+</sup> failed to support ligation. In comparison, ATP-dependent

10 20 30 40 50 60 70 80 90 100

ligase from Hin (i.e. *Haemophilus influenzae*) uses only  $Mg^{2+}$  and  $Mn^{2+}$  as the metal cofactor for nick closure but not  $Ca^{2+}$ ,  $Co^{2+}$ ,  $Cu^{2+}$ , and  $Zn^{2+}$  (Cheng, et al., *Nucleic Acids Res*, 25(7):1369-1374 (1997), which is hereby incorporated by reference); ATP-dependent ligase from *Chlorella* virus PBCV-1 can use  $Mg^{2+}$ ,  $Mn^{2+}$ , and  $Co^{2+}$  but not  $Ca^{2+}$ ,  $Cu^{2+}$ , and  $Zn^{2+}$  (Ho, et al., *J Virol*, 71(3):1931-1937 (1997), which is hereby incorporated by reference). Using  $Ca^{2+}$  as the metal cofactor, *Thermus* enzymes were able to convert most of the substrate into the DNA-adenylate intermediate. However, the rates of nick closure were reduced which led to the accumulation of the DNA-adenylate intermediate (Figure 4B). A small amount of the intermediate was observed with  $Ni^{2+}$ ; however, ligation product was not observed at the current detection level, suggesting that  $Ni^{2+}$  could not support the nick closure step (Figure 4B). To further compare the relative activity of the two *Thermus* ligases with  $Mg^{2+}$  and  $Mn^{2+}$ , the generation of ligation product was first monitored over a 20-min time period. As shown in Figure 5, the *Thermus* enzymes were consistently more active with  $Mg^{2+}$  than with  $Mn^{2+}$ . Second, ligation activity up to 40 mM  $Mg^{2+}$  or  $Mn^{2+}$  concentrations (Figure 6) was assayed. Both of the enzymes responded sensitively to the change of the metal ion concentration in the reaction mixture. At high  $M^{2+}$  concentrations, the high ionic strength may inhibit the enzyme activity, consistent with KCl dependence profile (Figure 4). Similar to the time-course results, the *Thermus* enzymes were more active with  $Mg^{2+}$  than with  $Mn^{2+}$  (Figure 6). The discrepancy on the relative activity of *Thermus* ligases between this study and an earlier report may be due to use here of cloned enzymes while the earlier work used purified native enzyme (Takahashi, et al., *J Biol Chem*, 259(16):10041-10047 (1984), which is hereby incorporated by reference).

25

#### Example 11 - Steady State Kinetics

The steady state kinetic constants were measured by monitoring the formation of fluorescently labeled ligation product over time using substrate concentrations spanning estimated  $K_m$  values (Table 1).

TOP SECRET - EQUITY 360

**Table 1.** Steady state kinetics of *Tth* and *Tsp.* AK16D ligase <sup>a</sup>

| Ligase            | <i>K<sub>m</sub></i> (nM) | <i>k<sub>cat</sub></i> (min <sup>-1</sup> ) | <i>k<sub>cat</sub>/K<sub>m</sub></i> (M <sup>-1</sup> s <sup>-1</sup> ) |
|-------------------|---------------------------|---|---|
| <i>Tth</i>        | 87                        | 56  | 1.1 x 10 <sup>7</sup>   |
| <i>Tsp.</i> AK16D | 104                       | 38  | 0.62 x 10 <sup>7</sup>  |

<sup>a</sup>: Results represent the average of at least three experiments.

5 The steady state properties of *Tsp.* AK16D ligase were similar to *Tth* ligase, indicating that the catalytic channels are highly conserved in *Thermus* ligases. The average *K<sub>m</sub>* value of about 90 nM for *Thermus* ligases is similar to the *K<sub>m</sub>* value of 50 nM for *E. coli* ligase (Modrich, et al., *J Biol Chem*, 248(21):7495-7501 (1973), which is hereby incorporated by reference) and about 10-fold higher than vaccinia 10 virus ATP-dependent ligase (Sekiguchi, et al., *Nucleic Acids Res*, 25(4):727-734 (1997), which is hereby incorporated by reference). The average *k<sub>cat</sub>* value of about 45 turnovers per min for *Thermus* ligases is higher than the *k<sub>cat</sub>* value of 28 turnovers per min for *E. coli* ligase (Modrich, et al., *J Biol Chem*, 248(21):7495-7501 (1973), which is hereby incorporated by reference).

15

#### **Example 12 - Ligation Of Gapped Or Inserted DNA Duplex Substrates**

Gapped substrates were formed by deleting one or two nt from the 3' hydroxyl site of oligonucleotide LP3'C, and inserted substrates were formed by 20 adding one or two nt at the 3' hydroxyl site of oligonucleotide LP3'C. Gapped or inserted duplexed DNA sequences are distinctively different from normal nicked substrate. Under our experimental conditions, no ligation was detectable with 1-nt (i.e. nucleotide) or 2-nt gapped or 2-nt insertion substrates for either *Tth* or *Tsp.* AK16D ligase (Figure 7A). As for 1-nt insertion substrates, only A insertion gave a 25 trace amount of ligated products for both ligases (Figure 7A). All other 1-nt insertions at the ligation junction could not be ligated. In contrast, Hin ligase and *Chlorella* ligase demonstrate observable ligation with 1-nt gap (Ho, et al., *J Virol*, 71(3):1931-1937 (1997) and Cheng, et al., *Nucleic Acids Res*, 25(7):1369-1374 (1997), which are hereby incorporated by reference). In the case of vaccinia ligase, 30 the ligation of 1-nt gap is negligible but the formation of DNA-adenylate intermediate is significant, suggesting the major impact of using 1-nt gapped substrate is on nick

closure (Shuman, S., *Biochemistry*, 34(49):16138-16147 (1995), which is hereby incorporated by reference). The formation of DNA-adenylate intermediate with the *Thermus* enzymes was not observed, suggesting that most of the gapped or inserted substrates may have abolished the possibility of completing the second step in the 5 ligation cycle — adenylation of DNA substrate at the 5' phosphate. The 1-nt A insertion mis-ligation could be due to slippage (Figure 7B). Although *Thermus* ligase slippage is far less than *Thermus* DNA polymerase, it does occur at a low frequency. Given the fact that the adjacent nt is a T, the slippage could have occurred at 5' 10 phosphate side where a 5'A/C mismatch is ligated (Luo, et al., *Nucleic Acids Res*, 24(15):3071-3078 (1996), which is hereby incorporated by reference). It is unlikely that the enzyme tolerates slippage on the 3' side, because a 1 nt C insertion did not 15 yield detectable ligation product (Figure 7).

#### Example 13 - *Thermus* DNA Ligase Fidelity

15 *Tth* DNA ligase is more discriminative when the mismatch is located at the 3' side of the nick junction. 3'G/T or 3'T/G is the only mismatch that shows observable mismatch ligation (Luo, et al., *Nucleic Acids Res*, 24(15):3071-3078 20 (1996), which is hereby incorporated by reference). To evaluate the fidelity of the cloned *Tsp*. AK16D ligase, the rate ratio of match over 3'T/G mismatch ligation was compared with wild-type and K294R mutant *Tth* DNA ligases along with T4 ligase from a commercial source (Table 2).

**Table 2.** DNA ligase fidelity <sup>a</sup>

| Ligase             | Enzyme Concentration (nM) | Initial rates of C-G match (fmol/min) | Initial rates of T-G mismatch at 3'-end (fmol/min) | Initial rates of T-G mismatch at penultimate 3'-end (fmol/min) | Ligation fidelity 1 <sup>b</sup> | Ligation fidelity 2 <sup>c</sup> |
|--------------------|---------------------------|---------------------------------------|--|--|----------------------------------|----------------------------------|
| T4                 | 0.5                       | $1.4 \times 10^2$                     | 2.8  | 7.1  | $5.0 \times 10^1$                | $1.9 \times 10^1$                |
| <i>Tth</i> -wt     | 1.25                      | $5.5 \times 10^1$                     | $6.5 \times 10^{-2}$                               | $2.9 \times 10^{-1}$   | $8.4 \times 10^2$                | $1.9 \times 10^2$                |
| <i>Tth</i> -K294R  | 12.5                      | $1.5 \times 10^2$                     | $2.3 \times 10^{-2}$                               | $4.3 \times 10^{-1}$   | $6.3 \times 10^3$                | $3.4 \times 10^2$                |
| <i>Tsp</i> . AK16D | 12.5                      | $1.3 \times 10^2$                     | $2.5 \times 10^{-2}$                               | $1.2 \times 10^{-1}$   | $5.1 \times 10^3$                | $1.1 \times 10^3$                |

<sup>a</sup> The reaction mixture consisted of 12.5 nM nicked DNA duplex substrates, indicated the amount of DNA ligases in ligation reaction buffer. T4 DNA ligase fidelity was assayed at 37 °C, thermophilic ligase fidelity was assayed at 65°C. Five  $\mu$ l Aliquots from a 160  $\mu$ l reaction mixture were removed at 0, 10, 20, 30, 40, 50, 60 s for reactions containing matched substrates and at 0, 1, 2, 3, 4, 5, 6 h for reactions containing mismatched substrates, and mixed with 5  $\mu$ l of stop solution. Samples (5  $\mu$ l) were electophoresed through an 8 M urea-10% polyacrylamide gel as described. Fluorescently labeled ligation products were analyzed and quantified using Genescan 672 version 2.0 software (Applied Biosystems, Foster City, CA). The results were plotted using DeltaGraph Pro3 software (DeltaPoint Inc., Monterey, CA). The initial rates were determined as the slope of linear range of the graph with the x-axis as the time and the y-axis as the amount of the ligation product generated. A schematic illustration of matched and mismatched substrates are as follows:

|   |   |   |
|---|---|---|
| C-G match at 3'-end   | T-G mismatch at 3'-end  | T-G mismatch at penultimate 3'-end  |
|  |  |  |

<sup>b</sup> Ligation fidelity 1= Initial Rate of C-G match / Initial Rate of T-G mismatch at 3'-end.

<sup>c</sup> Ligation fidelity 2= Initial Rate of C-G match / Initial Rate of T-G mismatch at penultimate 3'-end. The concentrations of DNA ligases used in each experiment are as indicated. Results were calculated as the average of at least two experiments.

25

T4 ligase demonstrated high catalytic efficiency toward both match and 3'T/G mismatch substrate such that a ligation fidelity of 50 was obtained. *Thermus* ligases appeared to be less efficient in match ligation as evidenced by the requirement of 30 higher enzyme concentration to achieve comparable match ligation rate. However, under the same assay conditions, *Thermus* enzymes were far less prone to ligate a 3'T/G mismatch. As a result, the fidelity of *Thermus* enzymes was 17- to 126-fold higher than T4 ligase (Table 2, Ligation fidelity 1). The fidelity of the newly cloned *Tsp.* AK16D ligase was similar to K294R *Tth* mutant but 6-fold higher than wild-type 35 *Tth* enzyme. A DNA-adenylate intermediate was observed with 3'T/G mismatch

ligation, suggesting that a mismatch at the 3' ligation junction imposes substantial constraints on the ability of *Thermus* ligases to close the nick, thereby limiting the turnover of DNA-adenylate intermediate into ligated product and free AMP (the third step of ligation cycle). The effects of moving the T/G mismatch one base-pair away  
5 from the ligation junction was further examined. The rates of ligation with a T/G mismatch at the penultimate 3' end in general improved several-fold as compared with the T/G mismatch at the 3' end of the ligation junction. However, the ligation rates were still much slower than those of match ligation, emphasizing the importance of nucleotide complementarity near the ligation junction as well as the ultimate critical  
10 role of the perfect base-pair at the 3' end in controlling ligation reaction.

Consequently, the ligation fidelity when the mismatch was at the second position from the 3' side (ligation fidelity 2) was lower than that when the mismatch was located immediately at the ligation junction. It is noteworthy that the *Tsp.* AK16D enzyme maintains extremely high fidelity ( $1.1 \times 10^3$ ) even when the mismatch is at  
15 the penultimate position, further underscoring the discriminative power of this new *Thermus* ligase.

#### Example 14 - Thermostable DNA Ligase Fidelity In The Presence Of Mn<sup>2+</sup>

20 Many enzymes such as DNA polymerase and restriction endonucleases demonstrate relaxed specificity when Mn<sup>2+</sup> is used as the metal cofactor. The influence of metal ion substitution on ligase fidelity has not been fully investigated although it is known that Mn<sup>2+</sup> can be used as an alternative metal cofactor for a ligation reaction ((Ho, et al., *J Virol*, 71(3):1931-1937 (1997) and Cheng, et al.,  
25 *Nucleic Acids Res*, 25(7):1369-1374 (1997), which are hereby incorporated by reference). The reaction rates of the match and mismatch ligation for *Tsp.* AK16D ligase and *Tth* ligase were determined. As shown in Table 3, the match ligation rates were higher with Mg<sup>2+</sup> than with Mn<sup>2+</sup> (Table 3), in agreement with the consistent high ligation rate under various Mg<sup>2+</sup> conditions (Figure 4-6).

**Table 3.** DNA ligase fidelity with  $Mn^{2+}$ <sup>a</sup>

| Ligase             | Concentration (nM) | Initial rate of C-G match (fmol/min) | Initial rate of T-G mismatch (fmol/min) | Ligation fidelity |
|--------------------|--------------------|--------------------------------------|---|-------------------|
| <i>Tth</i> -wt     | 1.25               | $2.6 \times 10^1$                    | $3.7 \times 10^{-1}$                    | $7.0 \times 10^1$ |
| <i>Tsp</i> . AK16D | 12.5               | $9.5 \times 10^1$                    | $1.1 \times 10^{-1}$                    | $8.6 \times 10^2$ |

<sup>a</sup> Reaction conditions were identical to those in Table 2, except that 10 mM Mn<sup>2+</sup> was used in place of Mg<sup>2+</sup>. Ligation fidelity was defined as the ratio of Initial Rate of C-G match divided by Initial Rate of T-G mismatch at 3'-end. Results were calculated as the average of at least two experiments.

10 The mismatch ligation rate of *Tth* ligase was about six-fold higher with Mn<sup>2+</sup> than with Mg<sup>2+</sup> while that of *Tsp*. AK16D ligase was about 4-fold higher. Thus, as with other previously studied DNA enzymes, DNA ligases also demonstrate relaxed specificity when Mg<sup>2+</sup> is substituted with Mn<sup>2+</sup>. As a result, the fidelity factors of *Tth* ligase and *Tsp*. AK16D ligase were reduced 12- and 6-fold, respectively (Tables 2-3).

15 Remarkably, the *Tsp*. AK16D enzyme retains 12-fold higher fidelity against mismatch ligation than the *Tth* enzyme. In contrast to using Mg<sup>2+</sup> as the metal cofactor, *Tth* ligase did not generate DNA-adenylate intermediate during 3'T/G mismatch ligation with Mn<sup>2+</sup>. This observation suggests that the nick closure of a 3'T/G mismatch by the *Tth* enzyme is accelerated with Mn<sup>2+</sup>. On the other hand, the *Tsp*. AK16D enzyme 20 accumulated DNA-adenylate intermediate during 3'T/G mismatch ligation with either Mg<sup>2+</sup> or Mn<sup>2+</sup>. These results indicate that the nick closure of a 3'T/G mismatch with Mn<sup>2+</sup> by *Tsp*. AK16D DNA ligase remains as the rate-limiting step, which accounts for the higher fidelity of this enzyme.

Studies on *Tth* DNA ligase has deepened understanding of thermostable ligases and has reaffirmed the common theme of ligation — adenylation of ligase at the KXDG motif (Luo, et al., *Nucleic Acids Res*, 24(15):3079-3085 (1996), which is hereby incorporated by reference). This study reveals that *Thermus* ligases may differ from each other as to substrate specificity despite their highly identical primary protein sequences. A highly homologous structure can be anticipated from various *Thermus* ligases, but subtle local environments may dictate the probability of accepting a particular mismatch as the substrate. The fidelity of the

*Thermus* ligases may be determined by multiple domains, multiple motifs and/or multiple sequence elements. In comparison of *Tth* and *Tsp*. AK16D ligases, one can find that although K294R (in an identical local environment, see Figure 1B) enhances the fidelity of *Tth* ligase (Luo, et al., *Nucleic Acids Res*, 24(15):3071-3078 (1996), 5 which is hereby incorporated by reference), *Tsp*. AK16D ligase with a K in this position can still demonstrate superior mismatch discrimination. Additional sequence elements remain to be uncovered. The R substitution at the adjacent position to the KXDG motif may have an effect on the *Tsp*. AK16D ligase's specificity, because studies on *Chlorella* ligase has emphasized the importance of occupying AMP 10 binding pocket for nick recognition (Sriskanda, et al., *Nucleic Acids Res*, 26(2):525-531 (1998)). The accumulation of DNA-adenylate intermediate with some divalent metal ions by *Tsp*. AK16D ligase asserts that the nick closure step of a ligation reaction can be sensitive to the selection of metal ions, gapped substrates and mismatch substrates. More structural and functional studies on *Tsp*. AK16D ligase 15 could reveal how this enzyme achieves high fidelity with different substrates and different metal ions.

Although the invention has been described in detail for the purpose of illustration, it is understood that such details are solely for that purpose and that variations can be made therein by those skilled in the art without departing from the 20 spirit of the scope of the invention which is defined by the following claims.

4350007-30025610